G. BARRY PIERCE, MD

The Cancer Cell and Its Control by the Embryo

Rous–Whipple Award Lecture

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THE GREAT HONOR that you have awarded me today was unanticipated, and the more treasured because the Association also honors Professor Rous and Professor Whipple, scientists for whom I have the greatest admiration. It is also a happy occasion because it allows me the privilege of acknowledging the men who trained me: H. E. Rawlinson and R. F. Shaner at the University of Alberta, and F. J. Dixon at the University of Pittsburgh. It is a pleasure to acknowledge the support of A. J. French during the struggle to become established; and R. H. Fennell Jr., at the University of Colorado has been a true friend and a constant source of strength and inspiration. If I had a brother, I'd like him to be just like Hank Fennell. Finally, I would like to say "Thank you" to the Fellows, Residents, and Students who have contributed so much to this work and brightened each day.

In this lecture I propose to discuss a concept of cancer that has been formulated upon our experiments on differentiation and cancer, outline the current experiments from our laboratory in regulation of cancer by the embryo, and indicate the potential importance of this approach for therapy.

Despite enormous successes in the treatment of certain tumors, after 30 years of intensive study, the conclusion is unavoidable that alternatives to cytotoxic therapy are desperately needed for the treatment of carcinoma with metastases. I would propose direction of differentiation of malignant to benign cells as the most promising alternative. This idea is based upon our demonstration in 1959 that embryonal carcinoma cells of testicular teratocarcinomas could spontaneously differentiate,[1] and in 1961 that these differentiations could be modulated *in vitro*.[2]

To refresh your memory, a teratocarcinoma is an extremely malignant tumor containing a heterogeneous collection of differentiated tissues representing each of the primary germ layers, plus embryonal carcinoma. When the embryonal carcinoma was separated from the other tissues and tested *in vivo*, it proved to be a multipotent stem cell tissue capable of forming the differentiated tissues of the teratocarcinoma.[1] The differentiated tissues proved to be benign.[3] These observations were confirmed in experiments performed in association with L. J. Kleinsmith in which embryonal carcinoma cells were successfully cloned *in vivo* in 1964.[4]

It was disheartening to see the equanimity with which these observations were received. The reason appeared to be that teratocarcinoma was considered an an oddity in oncology. As such, oncologists believed its behavior might be an exception to the general rules of oncology. Accordingly, over the next years I studied differentiation in squamous cell carcinoma,[5] chondrosarcoma,[6] and adenocarcinomas of the breast and colon[7] and demonstrated that the rules learned from teratocarcinoma governed the behavior of neoplasms in general. For example, the malignant cells of squamous cell carcinoma terminally differentiated into benign squamous cells incapable of forming a tumor.[5]

After the discovery by Bradley and Metcalf[8] and Pluznik and Sachs[9] in 1965 of colony-stimulating factors, which were essential for the growth of normal and leukemic cells *in vitro*, leukemologists became interested in differentiation. Clones of leukemic stem cells developed into colonies containing macrophages and granulocytes. Clearly, the lessons learned from teratocarcinoma could be applied to leukemia.

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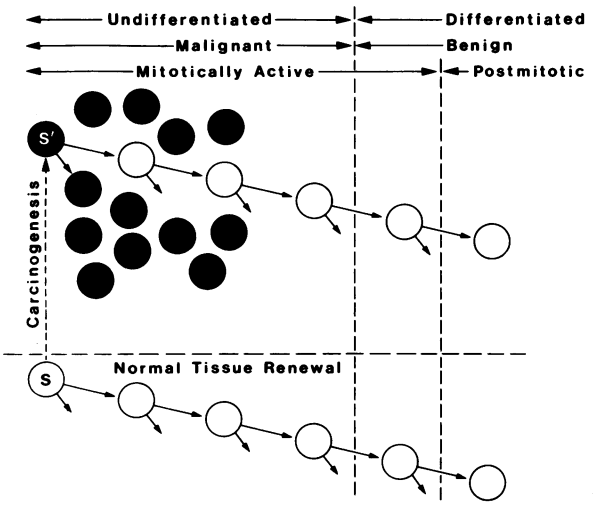

Figure 1—Concept of a tumor. The normal cell lineage of tissue renewal is at the *bottom*. The tumor is at the *top*. The caricature is formed by the number of malignant cells in relationship to the number that mature. For simplicity's sake the offspring of the malignant derivatives of S' have been left out of the figure.

During the 1960s, L. C. Stevens discovered that the primordial germ cell was the cell of origin of the embryonal carcinoma of the testis.[10] With Dr. Stevens, P. K. Nakane and I showed that the primordial germ cell was no more differentiated, from an ultrastructural standpoint, than the embryonal carcinoma cell to which it gave origin.[11] This meant that it was unnecessary to postulate dedifferentiation as a mechanism to explain the undifferentiated appearance of this carcinoma. Then, in later studies, the rule was established that the stem cells of normal tissues are no more differentiated than the malignant stem cells of their corresponding carcinomas.[7]

By 1974, enough data were available to formulate a useful concept of carcinoma, which is outlined in Figure 1. A carcinoma is a caricature of the normal process of tissue renewal.[12] The normal process of tissue renewal allows for replacement of precisely the number of cells that become senescent, as illustrated in the lower part of Figure 1. The malignant stem cell is derived from the normal stem cell, which it closely resembles,[7,11] but the controls of the neoplastic cell allow for the production of many malignant cells with differentiation of only a few of them, resulting in the undifferentiated appearance of the tumor.

A concept is only as good as it is useful. Among other things, this one is useful in understanding the origin of benign and malignant tumors as well as the histologic grade of tumors. For example, it is well known that both benign and malignant tumors arise during experimental viral[13] and chemical carcinogenesis.[14] The benign tumors develop first, an observation that led to the notion that benign tumors are a stage in the development of malignant tumors. This idea is compatible with the widely held notion of the monoclonal origin of most tumors. As an alternative, the model predicts that benign and malignant tumors arise from transformation of cells at different stages of differentiation (Figure 2). If this is true, then more than one cell would be involved in oncogenesis, and tumors that are multiclonal initially would become monoclonal by selection. The model also predicts that tumors that become malignant contain malignant cells from the outset; but because these cells are in subthreshold numbers for expressing their phenotype, tumors containing them initially behave in a benign manner.

The concept predicts that a benign tumor would be composed of C′ and D′ cells, illustrated in Figure 2. There is no evidence that C′ cells could give rise to S′ cells by dedifferentiation. C′ cells could arise by differentiation of all of the progeny of an S′ cell, but it is more probable that most C′ cells originate by transformation of the nearly terminally differentiated C cell, which it closely resembles (Figure 2). The resultant C′ cell is able to differentiate into D′ cells; and because it is minimally altered, it finds in the environment of the normal tissue the necessary ingredients to express its benign, neoplastic phenotype and form a mass. In contrast, an S′ cell, the stem cell of the malignant neoplasm, is markedly altered from normal and is incapable of quickly attaining the threshold number of cells required to express the malignant

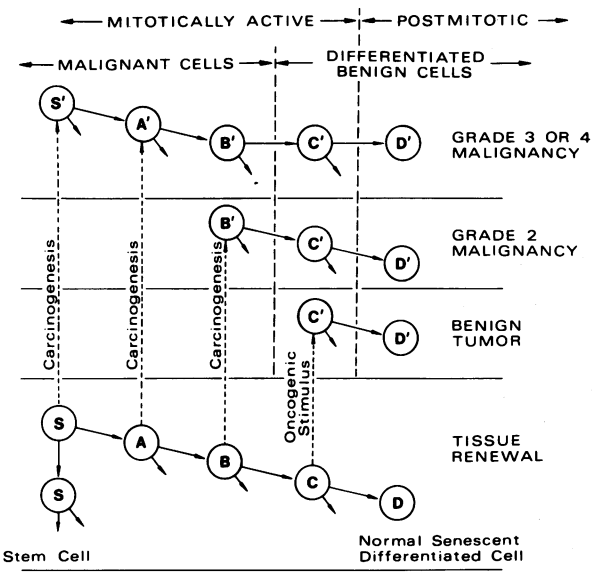

Figure 2—Differentiation and neoplasia. The normal cell lineage is at the *bottom*. Carcinogenesis may involve S, A, B, or C cells individually, giving origin to their corresponding malignant cell types. Their potentials for differentiation are indicated by the *arrows*. If S, A, B, and C cells responded to the carcinogenic insult simultaneously, the initial tumor would be as illustrated in Figure 3.

phenotype. The latent period is the time required for the S′ cell to achieve the critical mass required for phenotypic expression. It is well known from the studies of Grobstein and Zwilling in normal development that a threshold number of cells is required for the expression of a phenotype (as discussed in Andrews et al.[15]).

Carcinogenesis of A or B cells would result in malignant stem cells A′ or B′, each of which would resemble its normal counterpart and each of which would have its own potential for proliferation and differentiation, as illustrated in Figure 2. A tumor with B′ as its stem cell would be less malignant than one with A′ stem cells, for example. The leukemologists are now working upon this concept by attempting to show that antibodies to differentiated antigens of leukemias are really stage-specific differentiation antigens of the normal leukopoietic series. If an antigen is found in the B′ leukemic cells, for example, it would be expected to be present in the B cell.[16]

Only one *in vivo* situation is known in which initiated cells were easily identified microscopically before they produced a gross tumor. Stevens discovered that genital ridges of 12-day mouse embryos, when transplanted into the testes of adult mice, developed into fetal testes in which teratocarcinomas developed. The microscopic origin of the teratocarcinomas could be recognized in these fetal testes 7 days after transplantation of the genital ridges. Often only a single focus of tumor was found on serial section of a graft, but as many as 11 were observed in other specimens.[10] After 3 weeks a single large tumor was present in the testis. This observation proves that multiple normal cells may be transformed in a carcinogenic event, and monoclonality in this system would of necessity be the result of selection.

With this background, consider a cell lineage in which S and C cells (Figure 2) respond to an initiating event and produce an S′ and C′ cell. The C′ cell can outgrow the S′ cell in the normal environment, and after a few cell cycles there would be a preponderance of C′ and D′ cells in relation to the number of S′ cells. The tumor would be potentially malignant because it contains an S′ cell, but it would exhibit a benign phenotype because the S′ cells are in subthreshold numbers for expression of the malignant phenotype. This is exactly what Greene observed in his studies of spontaneously developing adenocarcinomas of the breast and uteri of rabbits. When small, the tumors did not metastasize and were not heterotransplantable, but when large they metastasized.[17] Clearly, as the hypothetical tumor grows, the S′ cells divide and eventually attain the threshold number required for them to express their phenotype. They have a rapid rate of growth, invade, and metastasize. The characteristics of the tumor change with selection of cells best able to survive, a phenomenon known as progression.[18] This is illustrated in Figures 3 and 4.

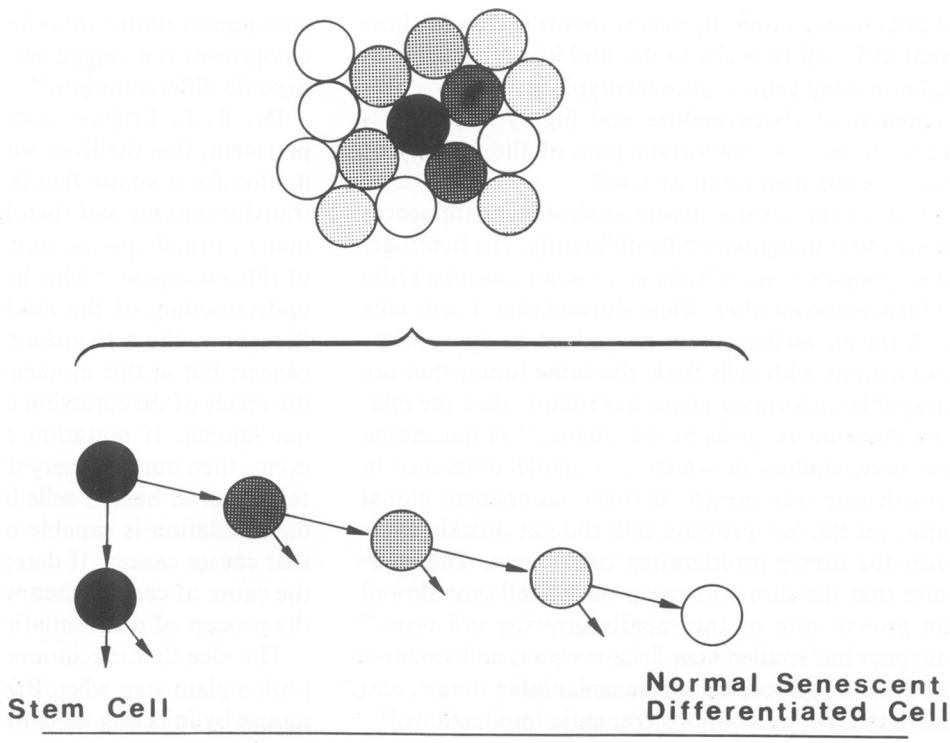

Figure 3 — Carcinogenesis. The normal cell lineage is at the *bottom* of the figure. The stem cell of the lineage is colored *black*. If it and each of its offspring capable of division responded to a carcinogenic insult simultaneously, the hypothetical tumor would appear as illustrated at the top. The tumor is potentially malignant, because it contains a malignant stem cell. That cell is in subthreshold number to express its phenotype, however. The white terminally differentiated cell of the normal lineage cannot undergo oncogenesis. The white cells in the tumor are derived by differentiation from proliferating cells. The *lighter-shaded* cells are least altered and initially divide more rapidly than the black cells because of their ability to exploit their environment. This tumor would behave in a benign manner.

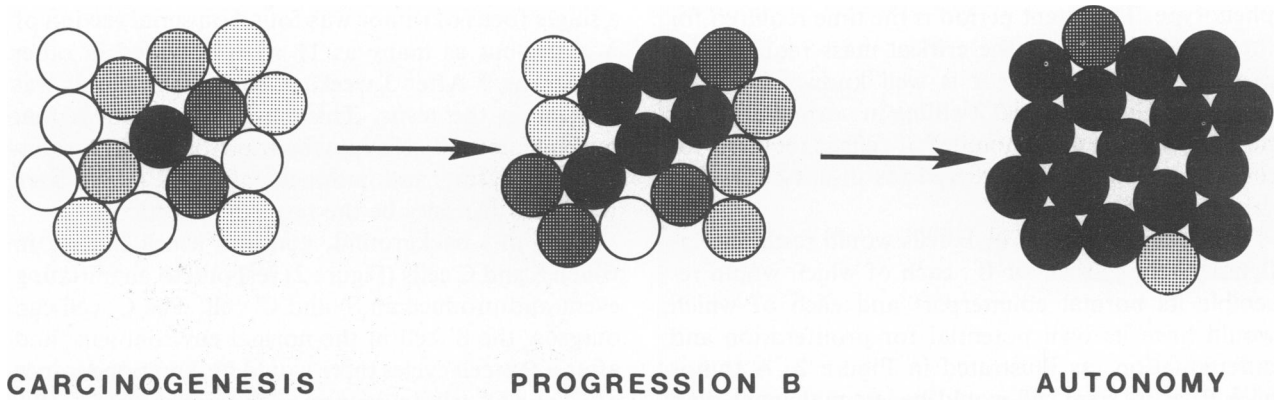

Figure 4—The tumor illustrated in Figure 3 is at the *left*. With progression, the cellular composition changes, with resultant changes in the phenotype of the tumor; eventually the tumor is autonomous (and apparently monoclonal in origin).

Repeated transplantation of tumors selects against those cells capable of differentiating, resulting thereby in more undifferentiated tumors (Figure 4). This process is complicated by the heterogeneity that can be superimposed upon the system by a variety of genetic events, including mutation and translocation. These genetically altered cells are also selected for survival of the fittest, and the properties of the tumors continue to change with time.[18] It is not known how often mutation or translocation happens in spontaneous tumors, but it is clear that it occurs frequently in tissue culture and probably quite often in highly selected transplantable tumors. Thus, "genetic instability of cancer" has become a catch phrase. It is worth remembering, however, that it is easy to show what cells can be made to do, and it is often difficult to know what cells do. Is karyotypic instability a phenomenon of tissue culture and highly selected tumors, or is it an important part of the biology of spontaneous neoplasms *in vivo*?

Just as the normal tissue environment influences benign and malignant cells differently, the heterogeneous populations of cells in a tumor can markedly influence one another. Klein showed that if cells able to form an ascites tumor are mixed in appropriate proportions with cells from the same tumor that are incapable of forming an ascites tumor, then the mixture does not form an ascites tumor.[19] A melanoma has been studied in which a fourfold difference in growth rate was present between component clonal lines; yet the fast-growing cells did not quickly overgrow the slowly proliferating component. This indicates that the slowly growing tumor cell type slowed the growth rate of the rapidly growing cell type.[20] Heppner has studied how drug-resistant and sensitive cells affect each other in a transplantable tumor, and the data have profound therapeutic implications.[21]

The arrow connecting the normal and malignant stem cell in Figure 1 implies a mechanism. Normal cells can be transformed to malignant cells with chemicals and viruses, or transformation may occur spontaneously. Most oncologists believe that insertion of viral information into the genome, or mutation (a structural change in the genome), is the underlying mechanism of carcinogenesis. On the basis of our experiences with spontaneously occurring embryonal carcinoma, and because all of the phenotypic traits of malignant cells appear to be encoded in the genome of normal cells, I favor the idea that the production of a neoplasm is probably similar to the production of any normal tissue.[22] The mechanism of tissue genesis involves cell division, differentiation, and organization.[15] In other words, I believe that carcinogenesis is an epigenetic event, similar to postembryonic differentiation.[22]

Dr. R. L. Erikson discovered,[23] while in our department, that the Rous sarcoma virus encodes information for a kinase that is responsible for malignant transformation, and then he discovered that cells of many normal species contain a cellular counterpart of this oncogene.[24] This holds great promise for our understanding of the mechanism of carcinogenesis. Somehow, the activation of this oncogene results in cancer; but at this moment the mechanism, whether the result of derepression or mutation of the gene, is not known. If mutation proves to be the causitive event, then our discovery that malignant cells can differentiate to benign cells implies that the process of differentiation is capable of regulating the mutation that causes cancer. If derepression of an oncogene is the cause of cancer, then what we have shown is that the process of differentiation represses the oncogene.

The idea that carcinoma cells could be reregulated took a giant step when Brinster produced a chimeric mouse by injecting an embryonal carcinoma cell into a blastocyst followed by transfer of the injected blas-

tocysts into the uteri of animals made pseudopregnant.[25] The chimeric mouse was recognized because it had coat colors representing the tumor and embryo strains. Clearly, the cancer cell injected into the blastocyst had been regulated and had taken part in normal embryonic development. This remarkable observation was quickly confirmed by two other laboratories.[26,27] We reasoned that if assays capable of yielding quantitative data could be developed, it should be possible to determine how the blastocyst regulates the cancer cell.[28]

The first assay to be developed was a tumor assay. It measured the effect of the blastocyst upon embryonal carcinoma cells by comparing the incidence of tumors obtained from single or small numbers of embryonal carcinoma cells injected into blastocysts, which were then injected into animals, with the incidence of tumors obtained when the same number of cells were injected into animals in the absence of blastocysts. The blastocysts employed were obtained 3½ days after observation of mating plugs. To refresh your memory, the blastocyst is a hollow, fluid-filled sphere 80 μ in diameter (Figure 5). It is enclosed by the zona pellucida, which is an amorphous layer that morphologically resembles basement membrane. Two cell types form the blastocyst: about 50 trophectodermal cells line the zona pellucida and enclose 12–14 inner cell mass cells. Trophectoderm forms the placenta; the inner cell mass, the embryo. The blastocoele contains 1×10^{-3} λ of fluid, which is pumped or secreted by the trophectoderm. The perivitelline space lies between the trophectoderm and the zona pellucida.

The techniques of producing injection chimeras were developed by Dr. Richard Gardner,[29] and I also employ some of the modifications of Dr. Clement Markert.[30] Briefly, embryonal carcinoma cells aspirated into a pipette of fine caliber are injected into a blastocyst held on a holding pipette by gentle suction. The injected blastocysts collapse, but after an hour's incubation they reexpand. Only reexpanded blastocysts with easily recognized embryonal carcinoma cells are employed in the assay. All operations are performed in a drop of media under oil with the use of a micromanipulator and a compound microscope.

The effect of the blastocyst upon three lines of embryonal carcinoma cells was tested. Tumor formation of F-9, a so-called nullipotent embryonal carcinoma, was not controlled; whereas that of 402A$\bar{x}$ and E.C. 247 was controlled.[31] Because of its ease of growth in tissue culture and its responsiveness to the blastocyst, E.C. 247 was chosen as the prototype tumor for the assay.

Because of the length of time required for the growth of the tumors and the inordinate expense involved, Dr. Robert Wells of our department developed an *in vitro* assay that was rapid and more economical.[32] This assay takes advantage of another neoplastic attribute of embryonal carcinoma. Tumor cells grow easily in tissue culture in comparison to normal cells. The assay measures the incidence of colonies of embryonal carcinoma cells grown *in vitro* alone or after incorporation into blastocysts. Briefly, blastocysts cultured *in vitro* hatch through the zona pellucida within 48 hours, and the protruding trophectoderm attaches to the substrate and differentiates into a patch of postmitotic trophoblast. A clump of inner cell mass cells overlies the trophoblast. If embryonal carcinoma cells are placed in the blastocoele and are controlled, typically no colony is formed. In the control situation, the embryonal carcinoma cells are placed in the perivitelline space, and at the time of hatching, grow and form colonies. These colonies grow continually until the endpoint of the assay, usually the sixth day.

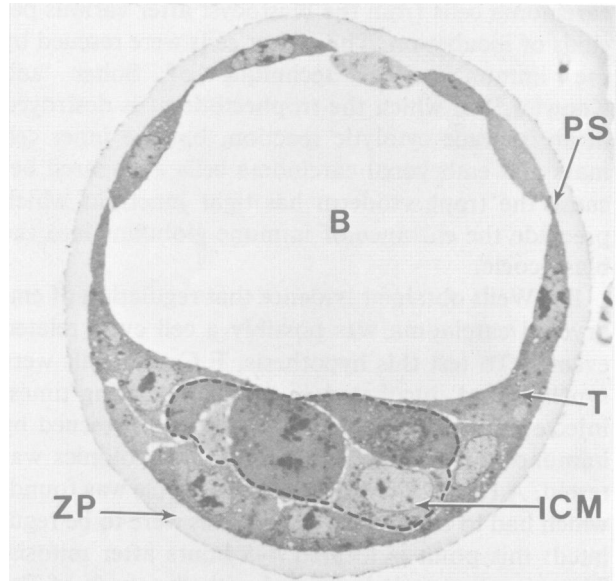

Figure 5—A blastocyst photographed with the scanning electron microscopy using backscatter imaging. This blastocyst is described in the text. *B*, blastocoele cavity; *PS*, perivitelline space; *T*, trophectoderm; *ICM*, inner cell mass; *ZP*, zona pellucida (×1000)

The assay indicated that colony formation of cells from E.C. 247 was regulated when the cancer cells were placed in the blastocoele, but they were not regulated when placed in the perivitelline space. It was concluded that the blastocyst could regulate tumor and colony formation of embryonal carcinoma cells.[28,31,32]

Dr. Wells employed this assay to determine the time required for the blastocysts to regulate colony formation of embryonal carcinoma cells.[32] This experiment required the successful rescue of embryonal

carcinoma cells from the blastocyst after various periods of incubation. The cancer cells were rescued by the immunosurgical technique of Solter and Knowles,[33] in which the trophectoderm is destroyed in an immune cytolytic reaction, but the inner cell mass and embryonal carcinoma cells are spared because the trophectoderm has tight junctions which preclude the entrance of immune globulins into the blastocoele.

Dr. Wells obtained evidence that regulation of embryonal carcinoma was possibly a cell cycle related event.[32] To test this hypothesis, E.C. 247 cells were synchronized, incubated *in vitro* for varying times, injected into blastocysts, incubated, and rescued by immunosurgery. Their ability to form colonies was tested. A restriction point in the cell cycle was found, which had to be traversed if the cells were to be regulated: this point is located 4–5 hours after mitosis. This observation is in accord with the work of Dr. Robert Scott,[34] who demonstrated a restriction point late in G-1 that cells must traverse if differentiation of adipocytes from fibroblasts is to occur.

We then turned to an analysis of the specificity of the blastocyst in the regulation of carcinoma. If the effect were nonspecific, then direction of differentiation could never be effective as an alternative to cytotoxic therapy.[31] To this end, L1210 leukemia and sarcoma 180 cells were tested in the blastocyst for control of tumor formation. Tumor formation was not controlled. This observation was in accord with the theory that only tumor cell types with a normal cellular counterpart in the blastocyst would be controlled by the blastocyst.

We also tested C1300 neuroblastoma, which lacks a normal counterpart in the blastocyst, and found it to be regulated to a small but reproducible and significant degree. It was postulated that the neuroblastoma was regulated by the neurula stage of development, which follows the blastula stage by about 4 days. This point will be discussed later. It was concluded that the mechanism of regulation of embryonal carcinoma by the blastocyst was reasonably specific.[31]

We next analyzed the mechanism whereby blastocysts regulate embryonal carcinoma cells. Regulation could be effected by diffusible molecules in the blastocoele fluid or by direct cell-to-cell contact. A micromicrocapillary tissue culture method was developed to test the effect of blastocoele fluid upon embryonal carcinoma cells.[35] Under the conditions of the experiment, the fluid was without inductive effect. To confirm this negative experiment, a situation was devised in which the embryonal carcinoma cell was bathed by blastocoele fluid but denied contact with the tissue culture media or with the cells of the blastocyst. After 24 hours of incubation in this milieu, the cancer cell was rescued for determination of whether it could produce a colony. The cancer cells grew colonies at control levels, indicating a lack of effect of blastocoele fluid under the conditions of the experiment. Thus, it was concluded by exclusion that direct cell-to-cell contact by the embryonal carcinoma cell with either trophectoderm or inner cell mass was necessary for the regulation of colony formation of the carcinoma cell.[35]

In testing the role of cell contact in regulation of colony formation, we isolated inner cell masses by immunosurgery, attached embryonal carcinoma cells to them, and found that under the conditions of the experiment, the inner cell masses were unable to regulate colony formation of embryonal carcinoma cells. By exclusion, it therefore appeared that colony formation of the embryonal carcinoma cell was regulated by direct cell-to-cell contact with trophectoderm.[35]

Because embryonal carcinoma cells in the perivitelline space are not regulated, it follows that the blastocoele surface of the trophectoderm must be the regulatory surface. The experiment examining this hypothesis was performed in the following manner. An embryonal carcinoma cell was injected into the blastocyst and placed on the blastocoele surface of the trophectoderm opposite the inner cell mass. The inner cell mass was amputated and removed by suction. In the process of amputation, the cut edges of the blastocyst sealed and formed a trophectodermal vesicle containing a cancer cell. The embryonal carcinoma cells were regulated in this situation.[35]

This is the point that we have reached with these studies. Interesting experiments lie ahead. We must determine whether the trophectoderm has surface molecules that regulate colony formation of the embryonal carcinoma cell, or whether the trophectodermal cells regulate the cancer cells via metabolic cooperation. We must also determine the fate of the embryonal carcinoma cells in the blastocysts. Some of the injected embryonal carcinoma cells are induced to differentiate because they form chimeras.[25–27] The embryonal carcinoma cells injected into blastocysts which form tumors obviously escape regulation. The fate of the balance of the embryonal carcinoma cells injected into the blastocyst is not known. Do these cells differentiate inappropriately, and are they thus unable to take part in chimera formation? Or are they destroyed by the blastocyst? As for our long-term goals, it does not matter which mechanism is

operative, either direction of differentiation or destruction of the cells, because the effect of the blastocyst is specific for embryonal carcinoma.

Because of the specificity of the control of embryonal carcinoma by the blastocyst, we wondered whether other embryonic fields might be able to regulate their closely related kinds of carcinoma. With Dr. A. Podesta, the low level of control of neuroblastoma that had been observed when neuroblastoma cells were injected in the blastocyst was examined.[36] To this end, 8½-day-old mouse embryos which were in early neurulation were injected in the second somite, and the embryos were cultured for 24 hours according to the methods of Beddington.[37] About 80% of these cultured embryos developed normally and had beating hearts, limb buds, and 20 or more pairs of somites. Only embryos judged to be normal were used in the experiments. The region of the second somite containing the neuroblastoma cells was dissected from the embryos and injected into the testes of A-strain mice. For the control, five neuroblastoma cells were injected into the testes of strain A mice alone or after they had been incorporated in a small fragment of liver tissue. Only one-sixth as many tumors were recovered from neuroblastoma cells that had been placed in the embryo in comparison to that of the controls.

Somites with their surrounding tissues and overlying ectoderm were dissected from the 8½-day-old mouse embryos, five neuroblastoma cells were then injected into them, and they were injected into the testes of A-strain animals. One-third fewer tumors were obtained in these experiments than in the control experiment, in which five neuroblastoma cells had been placed in the testis alone.[36] We are still awaiting the results of experiments to determine whether the regulation of tumor formation of neuroblastoma by the neurula is specific for neuroblastoma or not. Nevertheless, it would appear that the data justify the conclusion that just as the blastocyst can regulate embryonal carcinoma, the neurula can regulate tumor formation of neuroblastoma cells.

These observations are in accord with those of Gootwine, Webb, and Sachs, who injected leukemia cells into the placenta of 10-day-old mouse embryos and obtained mature animals with circulating leukocytes carrying leukemia cell markers.[38] This would suggest that the leukemia cells had been induced to differentiate by the embryos.

It is now clear that three embryonic fields can regulate their closely related malignant cell types, and thus it is our hypothesis that there must be an embryonic field capable of regulating every carcinoma. Since all tumors tested have some capacity for differentiation, and since even nullipotent embryonal carcinomas have been induced to differentiate with chemicals, study of how the embryo regulates malignant cells appears promising as an alternative to cytotoxic therapy for carcinoma.

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